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In Vivo Structures of the Helicobacter pylori cag Type IV Secretion System

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Report

**In Vivo Structures of the Helicobacter pylori cag Type IV Secretion System**

Graphical Abstract

**Highlights**

- *H. pylori* assemble unusual tube-like appendages when in proximity to gastric cells

- Architecture of the cag T4SS is remarkably similar to that of the *Legionella dot/icm* T4SS

- The cag T4SS cytoplasmic apparatus is likely a five-barrel structure

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**In Brief**

Bacteria assemble specialized type IV secretion systems (T4SSs) to inject molecular cargo into target cells. Using electron cryotomography, Chang and Shaffer et al. report the first structure of a cancer-associated T4SS in vivo and describe unique membranous appendages that are produced when *H. pylori* encounters gastric epithelial cells.

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**In Vivo Structures of the Helicobacter pylori cag Type IV Secretion System**

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**SUMMARY**

The type IV secretion system (T4SS) is a versatile nanomachine that translocates diverse effector molecules between microbes and into eukaryotic cells. Here, using electron cryotomography, we reveal the molecular architecture of the *Helicobacter pylori* cag T4SS. Although most components are unique to *H. pylori*, the cag T4SS exhibits remarkable architectural similarity to other T4SSs. Our images revealed that, when *H. pylori* encounters host cells, the bacterium elaborates membranous tubes perforated by lateral ports. Sub-tomogram averaging of the cag T4SS machinery revealed periplasmic densities associated with the outer membrane, a central stalk, and peripheral wing-like densities. Additionally, we resolved pilus-like rod structures extending from the cag T4SS into the inner membrane, as well as densities within the cytoplasmic apparatus corresponding to a short central barrel surrounded by four longer barrels. Collectively, these studies reveal the structure of a dynamic molecular machine that evolved to function in the human gastric niche.

**INTRODUCTION**

The type IV secretion system (T4SS) is a remarkably versatile molecular machine present in nearly all bacterial phyla and some archaeal species (Christie et al., 2005). Bacteria utilize T4SSs to interact with prokaryotic and eukaryotic cells and to export an incredibly diverse repertoire of substrates (Cascales and Christie, 2003). In most cases, T4SS activity is contact dependent and results in the delivery of nucleoprotein complexes and protein effectors directly into the target cell cytoplasm. By facilitating the exchange of genes and proteins among microbial populations and across kingdoms of life, the T4SS has accelerated bacterial evolution and resulted in species that thrive in diverse environments, including within plant and animal hosts (Christie et al., 2005; Walden et al., 2010). The gastric bacterium *Helicobacter pylori* exploits the cag-pathogenicity-island (cagPAI)-encoded T4SS (cag T4SS) to translocate a variety of effector molecules into gastric epithelial cells, including the oncoprotein CagA and fragments of peptidoglycan (Fischer, 2011). These translocated effector molecules activate components of the innate immune system and dysregulate signaling pathways that significantly augment the risk of gastric cancer (Amieva and Peek, 2016). When *H. pylori* contacts the gastric cell, the bacterium produces filamentous structures that are dependent on multiple cag genes and have been termed cag T4SS pili (Barrozo et al., 2013; Jimenez-Soto et al., 2009; Johnson et al., 2014; Kwok et al., 2007; Rohde et al., 2003; Shaffer et al., 2011). While *H. pylori* strains that fail to produce cag T4SS pili are unable to translocate cargo to host cells (Johnson et al., 2014; Kwok et al., 2007; Shaffer et al., 2011), the exact role of these filaments in cag T4SS activity remains unclear.

The T4SS family is phylogenetically diverse and has been divided into two major sub-types, type IVA and type IVB (T4ASS and T4BSS, respectively). Historically, T4ASSs have been classified according to protein homology to components of *E. coli* tra DNA conjugation systems (types F and P) and the *A. tumefaciens* vir T4SS, while T4BSSs exhibit protein sequence conservation to IncI-like conjugation systems and the *L. pneumophila* dot/icm T4SS (Wallden et al., 2010). In most cases, T4ASSs comprise approximately 12 components with clear homology to Vir proteins, while T4BSSs incorporate many more proteins, and few share sequence homology with vir T4SS components (Wallden et al., 2010). The cag T4SS has been considered a T4ASS since several Cag proteins share limited sequence similarity to Vir components (Fischer, 2011; Walden et al., 2010); however, the homologies are so weak that their relevance is unclear, and the cagPAI encodes as many genes as a typical T4BSS, including many *H. pylori*-specific genes (Fischer, 2011). In addition, recent work has provided architectural and structural information about several different T4SSs, including the *Escherichia coli* conjugation tra T4SS (Fronzes et al., 2009; Low et al., 2014), the *A. tumefaciens* vir T4SS (Gordon et al., 2017), the *Legionella pneumophila* dot/icm T4SS (Ghosal et al., 2017; Kubori et al., 2014), and the *H. pylori* cag T4SS (Frick-Cheng et al., 2016). Among these, the dot/icm and cag effector translocator systems have many more genes than the tra and vir DNA-translocating systems, including several...
without obvious homologs in other bacteria (Frick-Cheng et al., 2016; Ghosal et al., 2017; Kubori et al., 2014). A so-called “core complex” of the cag T4SS has been purified and consists of Cag3, CagT, CagM, and two constituents that are orthologous to the VirB9/TraO (CagX) and VirB10/TraF (CagY) subunits of other systems (Fischer, 2011; Frick-Cheng et al., 2016).

In the present study, we applied electron cryotomography (ECT) to image frozen-hydrated H. pylori co-cultured with human gastric epithelial cells. We report structures of the intact cag T4SS in vivo and describe membranous tubes elaborated by H. pylori in response to the host cell. Together, these results suggest hypotheses underlying the mechanism of the cag T4SS and the roles of its components.

RESULTS

H. pylori Cells Produce Membranous Tube-like Appendages in Response to the Host Cell

Since T4SS activity is stimulated by direct host cell contact, we sought to visualize the cag T4SS by imaging H. pylori in co-culture with human gastric epithelial cells. In order to avoid interference from flagella or flagellar motors in the analysis, we selected the cagPAI-positive, non-flagellated H. pylori strain 26695 for our studies. We cultured gastric epithelial cells on electron microscopy grids, infected the grid-adherent monolayers with H. pylori, and plunge-froze the co-culture sample to preserve cellular features in a near-native state. We recorded ECT tilt-series of regions of the sample where the bacteria were in direct contact or close proximity to epithelial cell elongations (Figures 1A and 1B). In approximately 5% of the tomograms, we observed striking membranous tubes extending from the outer membrane of wild-type (WT) H. pylori cells (Figures 1C and 1D). These tubes were produced only by H. pylori that were co-cultured with gastric cells and were not observed in bacteria that were grown in pure culture (n = 464; Figure S1A). While this indicates that the tubes assemble in response to the host cell, we did not observe direct interaction of individual tubes with gastric epithelial cell surfaces (though we cannot rule out the possibility that longer tubes touched host cell surfaces beyond the imaging area of individual tomograms). Densities possibly attributable to lipopolysaccharide (LPS) were clearly visible on the periphery of tube cross-sections, as were both leaflets of the membrane bilayer (Figure 1D, inset). A thin layer of periodic densities lined the interior of the tubes, intimately associated with the inner leaflet of the bilayer, suggesting the presence of a regular protein support scaffold (Figure 1E). The tubes appeared rigid and had membrane outer surface (not including LPS) and inner channel (interior surface of scaffold) diameters of 37 and 22 nm, respectively (Figure S2). Many tubes displayed pipe-like ports (median diameter, 10 nm) along their lengths (Figures 1F–1I). In some cases, ports appeared to induce small bends in the tube (Figure 1J), as if by wedging into the scaffold. The length of individual tubes produced by WT H. pylori ranged from 76 to 547 nm, with a median of 193 nm (n = 18; Figure S1B). To our surprise, the tubes were not associated with obvious basal body-like densities localized directly beneath the tube in the periplasm or the inner or outer membranes, suggesting that a dedicated membrane-bound apparatus either is not required for tube formation, not recognizable at the current resolution, or had disassembled prior to sample freezing.

To investigate whether the tubes were related to cag T4SS activity, we used ECT to image H. pylori lacking either the effector protein CagA, the cag T4SS plus-regulating protein CagH (Shaffer et al., 2011), or the entire cagPAI. ECT revealed tubes extending from the bacterial envelope when either cagA or cagH mutants were co-cultured with gastric cells (Figure S2), but not the cagPAI strain. Collectively, tubes were produced by H. pylori proximal to a gastric cell in a total of 17 of 336 tomograms of the WT, cagA, and cagH strains. We visualized roughly equivalent numbers of tubes produced by WT, cagA, and cagH strains (cagA, n = 22; cagH, n = 23), and the number of tubes produced per bacterial cell ranged from 1 to 10. Two extremely long tubes (785 nm and 1311 nm) were observed in the cagH mutant (Figure S1F), and this strain also assembled a few tubes with larger outer and inner diameter dimensions (Figure S1).

In Vivo Ultrastructure of the cag T4SS

In some tomograms, we noticed dense, periplasmic, cone-shaped particles spanning the bacterial envelope near membrane tubes (Figures 2A and 2B). These structures were reminiscent of L. pneumophila dot/icm T4SS complexes observed in situ (Ghosal et al., 2017) and consisted of distinct layers of densities in the periplasmic space near the outer membrane (Figure 2B). Based on the structural similarity to the dot/icm T4SS,
we hypothesized that these structures corresponded to a T4SS. Close inspection of our tomograms revealed varying numbers of these particles in each *H. pylori* cell (ranging from 0 to 4 particles per cell in the field of view of the tomograms). The particles were found at cell poles and lateral cell surfaces, consistent with previous reports analyzing cag T4SS components (Barrozo et al., 2018).
In many instances, we observed the bacterial outer membrane bulging to accommodate the assembled particle. We also captured several top views of the structure, which revealed two concentric rings. The outer ring exhibited 14-fold symmetry (Figures S2A, S2B, and S2D) and a diameter of 40 nm (Figures 2C and 2D), consistent with the structure of immunopurified cagT4SS core complexes resolved by negative-stain electron microscopy (Frick-Cheng et al., 2016). Among the four potential T4SSs harbored by H. pylori, the imaged strain lacks complete tfs3 and tfs4 systems, and although the strain harbors the comB T4SS, corresponding cone-shaped particles were never observed in over 100 tomograms of the cognate cagPAI mutant strain co-cultured with gastric epithelial cells, leading us to conclude that these particles are the cag T4SSs rather than the comB DNA uptake or other T4SSs.

To investigate structural details of the cag T4SS, we sought to generate a sub-tomogram average. Given the inherent structural flexibility of other T4SSs (Ghosal et al., 2017; Low et al., 2014), we aligned and averaged the periplasmic and cytoplasmic regions separately and then generated a composite average (Figure 2E). In total, 67 cag T4SS particles were captured and used to generate the average. Determined through Fourier shell correlation, the final resolutions of the periplasmic and cytoplasmic parts were 3.9 nm and 6.8 nm, respectively (Figures S2C and S2D). In the periplasm, we resolved a “hat” density associated with the outer membrane, several ring-like densities surrounding and beneath the hat, a central stalk, and wing-like densities on the periphery (Figures 2E, 2I, and 2J). Cross-sections through the cytoplasmic apparatus revealed parallel lines of density (Figures 2E–2H), but because most of the cag T4SS particles used in the average were imaged from the side (electron beam parallel to the membranes), the average was smeared by the missing wedge effect in that direction. Therefore, to interpret these densities, we explored a variety of candidate structures by generating artificial tomograms smeared by the same missing wedge effect and then compared their cross-sections to the experimental data (Figures S2E–S2H). We tested configurations of one to six barrel densities and various combinations of barrel and rod structures. The best matching model consisted of a short central barrel surrounded by four longer barrels, which together recapitulated the experimental results (Figures 2E–2H, 2K–2P; Figure S2).

Sheathed Cytoplasmic Rod within the cag T4SS
In three unusual cag T4SS particles (out of a total of 70 particles), we observed a dense, central rod extending from the outer-membrane-associated complex into inner membrane invaginations with different depths (Figures 3B–3D, Figure S2). This feature was not observed previously in the dot/icm T4SS or in the purified R388 T4SS particles (Ghosal et al., 2017; Low et al., 2014). The rod and inner membrane invaginations measured ~10 nm and 30 nm in width, respectively, and the rod extended 45–120 nm from the outer membrane (Figures 3B–3D). Notably, in one of the particles, the rod appeared to project through the inner membrane into the cytoplasm, though the details of this event were obscured by the crowded cytoplasm.

Comparison to Previous T4SS Structures
To explore the structural relationships between T4SSs, we compared the cag T4SS sub-tomogram average to the previous...
electron microscopy (EM) structures of purified sub-complexes of the R388 (Low et al., 2014) (Figures 4A and 4B), negatively stained sub-complexes of the cag T4SS (Figures 4C and 4D), and the sub-tomogram average of the in vivo dot/icm T4SS (Ghosal et al., 2017) (Figure 4E; Figure S3). In comparison to the R388 structures, the cag T4SS is similar in that it includes a large cluster of densities associated with the outer membrane, a stalk that connects the outer-membrane-associated cap to the inner membrane, and structures in the cytoplasm anchored to the inner membrane (Figures 4A and 4G). The size and shape of the R388 outer-membrane-associated cluster (referred to in Fronzes et al. [2009] as the “core complex” containing the C termini of VirB7, VirB9, and VirB10) matched the hat and inner ring density (labeled d in Figure S3E) in the cag T4SS structure (Figures 4C, 4D, 4G, 4H, orange demarcation). Because the CagY C terminus shares homology with VirB10 (Fischer, 2011; Rohde et al., 2003) and CagX shares homology with VirB9 (Fischer, 2011; Tanaka et al., 2003), we reason that CagX and CagY form the hat and the density labeled d. The size of the stalk and the configuration of the cytoplasmic barrels in the two structures, however, appear to be quite different.

In comparison to the images of the purified cag T4SS sub-complex that comprise CagM, CagT, CagX/VirB9, CagY/VirB10, and Cag3 (Figures 4C and 4G, blue demarcation), the periplasmic portion of the in vivo cag T4SS average exhibited almost the exact size and general shape, which allowed us to definitively position and orient the negative-stain result relative to the bacterial envelope. Comparison of the particle top views also revealed striking structural similarities, including in the sizes of the concentric rings and their 14-fold symmetry (Figures 4D and 4H). These observations further confirm that the particles averaged in this study are the cag T4SSs. From this comparison, and based on previous experimental evidence (Frick-Cheng et al., 2016), we conclude that CagT, Cag3, and CagM must produce the densities inside the blue outline but outside the orange in Figure 4G (which were already assigned to CagX/VirB9 and CagY/VirB10). CagT and Cag3 can be further pinpointed as the upper and lower outer rings (a and b in Figure S3E) based on the published negative-stain images of the CagX, CagY, and CagM sub-complex (Frick-Cheng et al., 2016), which are missing those rings (though which form the upper and the lower rings or, if they are mixed, remain unclear). By elimination, this suggests that CagM produces the density labeled g in Figure S3E; however, CagM localization could be more complicated and so remains to be verified.

Compared to the in vivo dot/icm T4SS structure, the cag T4SS structure is remarkably similar, considering the fact that the systems each comprise over 25 components and only a few share
sequence homology (Figure S3G). Both structures exhibit (1) an outer-membrane associated hat; (2) upper and lower outer ring-like densities surrounding the hat (labeled $\alpha$ and $\beta$ in Figures S3E and S3F); (3) a barrel-like $\gamma$ density at the center of the structure; (4) a central stalk; (5) weak, wing-like densities that extend from the inner membrane into the periplasm, and (6) parallel elongated densities perpendicular to the membrane in the cytoplasm (Figures S3C–S3F). While the upper and outer ring densities ($\alpha$) superimpose well (Figure 4G, right panel), a difference is that there are two densities in the lower ring of the cag T4SS (labeled $\beta$ and $\delta$ in Figure S3E) versus only one (labeled $\beta$ in Figure S3F) in the dot/icm T4SS. Based on a recent report describing the structure of the L. pneumophila dot/icm coupling protein DotL (Kwak et al., 2017), its predicted position within the secretion system just underneath the inner membrane (IM) (Kwak et al., 2017), and its match in size and shape to the central barrel seen at the same location in the H. pylori sub-tomogram average, we speculate that the central barrel of the cag T4SS cytoplasmic density corresponds to the Cag5/VirD4 coupling protein (Figures 4F and 4G, magenta demarcation; Figure S3H). The tentative positioning of Cag5 to the central barrel density of the cytoplasmic apparatus is further supported by recent work demonstrating that the VirD4 coupling protein is situated in the center of the R388 IM complex between VirB4 barrels (Redzej et al., 2017). Future work imaging deletion mutants will be needed to confirm or refute this speculation. Collectively, these data reveal that, although the cag T4SS is phylogenetically distinct from both the R388 and the dot/icm T4SS, the architecture of these three T4SS machines is remarkably conserved.

**DISCUSSION**

Here, we have reported the structure of the cag T4SS and shown that, when *H. pylori* are in proximity to host cells, the bacterium produces membranous tubes decorated with pipe-like ports. Multiple scanning electron microscopy (SEM) studies have shown that, under similar conditions, *H. pylori* assembles extracellular filaments (Jimenez-Soto et al., 2009; Johnson et al., 2014; Kwok et al., 2007; Rohde et al., 2003; Shaffer et al., 2011). In previous papers, these structures have been referred to as “pili,” “cag T4SS-associated pili,” “filaments,” “extensions,” etc., but here we will refer to all of the previously observed structures as “SEM filaments” for clarity, also because we would like to use the word “pilus” for a single component of the structure (the rod) described later. We propose that the membrane tubes visualized by ECT are the native form of the previously described SEM filaments for the following reasons. First, both the membrane tubes and SEM filaments were only observed when *H. pylori* was co-cultured with gastric epithelial cells. Second, in all cases, both structures depended on the presence of the genes in the cagPAI. Third, one previous study interpreted the SEM filaments as proteinaceous pili covered by membrane sheaths (Rohde et al., 2003). Fourth, and most decisively, deletion of the CagH “molecular ruler” protein resulted in both longer and wider membrane tubes and longer and wider SEM filaments (Shaffer et al., 2011). Concerning size, unfortunately, the different SEM studies reported substantially different diameters for the SEM filaments, ranging from 15 to 70 nm (Barrozo et al., 2013; Johnson et al., 2014; Kwok et al., 2007; Rohde et al., 2003; Shaffer et al., 2011). While this range does include the diameter of the native membrane tubes measured here, we speculate that, in the previous SEM studies, the extensive chemical fixation, dehydration, and metal coating inherent to the method may have introduced the variations.

Previous SEM immunolabeling experiments showed that CagY is present along the SEM filaments (Jimenez-Soto et al., 2009; Rohde et al., 2003) and that CagT is clustered in ring-like formations at the SEM filament base (Rohde et al., 2003). Other studies showed that additional Cag proteins could be localized to the SEM filaments, including CagA, CagL, CagT, and CagX (Barrozo et al., 2013; Jimenez-Soto et al., 2009; Kwok et al., 2007; Rohde et al., 2003; Tanaka et al., 2003). Because both the cag T4SS core complex and SEM filaments/membrane tubes have been associated with CagY, CagT, and CagX, we propose that the cag T4SS and the tubes are different states of the same secretion apparatus. In support of this, we note that the outer and inner diameters of the membrane tubes (37 and 22 nm, respectively) approximately match the outer and inner ring dimensions of the cag T4SS (41 and 19 nm, respectively). Alternatively, the membrane tubes may be a different part or assembly of the cag T4SS.

More specifically, we propose as a working hypothesis that the T4SS structure shown here (Figure 2E) is a “pre-extension” state that assembles in response to the presence of a host cell (Figure S4A). It is known that the *E. coli* traA and *A. tumefaciens* vir T4SSs produce protein pili. In the tra system, the protein F pilus has an outer diameter of 8.7 nm and is formed by the major pilin TraA/VirB2, which is otherwise found in the IM (Costa et al., 2016). Our data reveal that the cag T4SS also produces a rod-like structure with a similar diameter in the periplasm (Figure 3). Therefore, we propose that the cag T4SS also assembles a protein pilus (the rod) from subunits in the IM (Figures S4B and S4C). This pilus may be formed of CagC, which exhibits weak homology with the VirB2/traA component in the vir T4SS (Andrzejewska et al., 2006; Johnson et al., 2014). In our working model, we propose that, as the pilus grows upward from the IM, it interacts with components of the core complex within the periplasm, possibly opening a translocation channel through the system (Christie et al., 2005). At the onset of membrane tube formation, a conformational change within the core complex disengages the CagX/CagY hat, which is released from the CagT/Cag3 outer ring, and extended tubes are produced by growth of the thin protein scaffold lining the inside tube surface (Figure S4C). The tubes could be stabilized by the CagT/Cag3 outer ring, which remains at the base like a collar, and the scaffold, which holds their diameter constant along their length. This scaffold may contain the VirB5-like CagL (Kwok et al., 2007; Shaffer et al., 2011). If some part of CagL extended through the outer leaflet of the tube lipid bilayer, it would explain the observations that CagL can bind host cell integrins and can be localized to the SEM filaments by immunogold labeling (Kwok et al., 2007). CagL, which can also bind integrins (Jimenez-Soto et al., 2009), may also be part of the scaffold (Pham et al., 2012; Shaffer et al., 2011). This model would explain why CagL and CagH deletion mutants have no SEM filaments (Shaffer et al., 2011). Given that CagH is membrane bound, plays an essential role in T4SS activity,
regulates SEM filament/tube dimensions, and forms a complex with CagL and CagI (Shaffer et al., 2011), CagH may control the incorporation of CagL and CagI into the scaffold (Pham et al., 2012; Shaffer et al., 2011).

We propose that the CagX/CagY hat is a membrane-fusion machine positioned within the tubes. We propose that, upon contact with a host cell, CagX/CagY fuses the tube and host membranes, opening a channel for the passage of effectors (Figure S4D). Our interpretation of the tube ports is that they are open CagX/CagY channels, and this may explain the immuno-labeling results that CagY can be localized along the length of the SEM filaments (Jimenez-Soto et al., 2009; Rohde et al., 2003), as well as the observation that the C-terminal/VirB10 domain region of CagY can bind host integrins (Jimenez-Soto et al., 2009). While one CagX/CagY complex may be positioned at the tip of the tube, other complexes present in the outer membrane (OM) may be drawn upward into the tube as it extends, or, alternatively, additional CagX/CagY complexes may assemble in the tubes’ lateral walls after extension. The interpretation that CagY is the membrane-fusion protein could explain why strains lacking cagY form SEM filaments but do not secrete effector molecules (Barrozo et al., 2013; Johnson et al., 2014), and it is consistent with the proposal that CagY serves as a molecular switch that regulates secretion activity in vivo (Barrozo et al., 2013). In the imaged strains, the lateral ports exhibited a diameter of ~10–12 nm, which is large enough to facilitate transport of the folded CagA effector protein, whose dimensions measure 8 × 11 × 5.5 nm (Hayashi et al., 2012). One problem with this model is what happens to the IM transmembrane domain of CagY; given its tether to the IM, how would it remain at the tip of the extending tube? One possibility is that, as the IM is perturbed by pilin subunits being loaded out of the IM and onto the base of the pilus, and as the pilus itself may incorporate IM lipids similar to F pili (Costa et al., 2016), the CagY tether is somehow released. Another possibility is that, as CagY is ~1,900 amino acids long, it spans the length of the tube.

Our interpretation of the three unusual T4SS particles with pili protruding downward toward the cytoplasm (Figure 3) is that these are stalled end states in which the CagX/CagY hat has failed to disengage the CagT/Cag3 ring, forcing pilus growth to push the IM downward instead of the outer membrane upward. It may, therefore, be that the only states captured in cryotomograms are long lived, including the pre-extension state (Figure S4A), various stalled “failure-to-fire” states (Figure S4C), and terminal end states of tubes no longer connected to the host, leaving the tubes to reseal at the tip (Figure S4E). Future studies will focus on earlier stages of the association and on bacteria directly touching host cells, in hopes of producing images of the hypothesized transitory states (Figures S4B–S4D).

The role and fate of the pilus itself remains particularly unclear. While pilus growth might be involved in tube extension, a recent study revealed that SEM filaments can be produced by a strain lacking cagC (Johnson et al., 2014), and it is also known that strains lacking cagC do not secrete cag T4SS effectors (Andrezjewska et al., 2006; Johnson et al., 2014). Perhaps there is some required interaction between the putative CagC pilus and the core complex that primes the machinery for effector translocation. Alternatively, as proposed for VirB2 in other type IVA systems (Christie et al., 2005), CagC may form a stable, rod-like translocation channel or pore through the periplasmic core complex into the base of the tube when the system is actively secreting effector molecules. We emphasize that these speculations are simply our current working hypotheses, and much further work will be needed to confirm or refute this model.

Assuming our model is correct, the H. pylori cag T4SS differs from the E. coli tra or A. tumefaciens vir systems in that the cag T4SS produces an extracellular appendage enclosed by outer membrane. Perhaps all T4SSs will share the basic central machinery that loads a VirB9/VirB10 membrane-fusion machine at the tip of an appendage (pilus or tube) that then extends to open a channel into a host cell, but perhaps they will also differ in the presence and roles of peripheral proteins that manage the outer membrane involvement in that appendage. Functionally, exposed protein pilus may, alone, be sufficient to translocate single-stranded DNA (Shu et al., 2008), but wide membrane tubes like those seen here may be required to translocate folded effector proteins into a host. Interestingly, some authors have proposed that membrane tubes are involved in DNA translocation (Anderson, 1958; Anderson et al., 1957).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**

*H. pylori* strain 26695 and corresponding mutants (Shaffer et al., 2011) were routinely maintained on Trypticase soy agar supplemented with 5% sheep blood (BD Biosciences) under microaerobic conditions. For all experiments, *H. pylori* cells were seeded into Brucella broth supplemented with 10% fetal bovine serum (FBS) and were grown overnight in shaking culture at 37°C, 5% CO2. Overnight bacterial cultures were normalized to an optical density at 600 nm (OD600) to ~0.3 in fresh Brucella broth supplemented with 10% FBS, and cells were grown to mid-log phase at 37°C, 5% CO2, prior to generating samples for microscopy analysis.

**Human Cell Culture**

The gastric adenocarcinoma cell line AGS (ATCC CRL-1739) was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 10 mM HEPES buffer. Cells were grown at 37°C in 5% CO2.

**Electron Cryotomography**

Detailed methods of electron cryotomography sample preparation, data collection, and data processing are outlined in the Supplemental Experimental Procedures.

**Statistical Analysis**

The statistical analysis was undertaken in GraphPad Prism 7 by one-way ANOVA, followed by Dunnett’s multiple comparisons test.

**DATA AND SOFTWARE AVAILABILITY**

The accession numbers for the sub-tomogram averages of cag T4SS machinery that support the findings of this study are EMDB: 7474 (aligned on the periplasmic region) and EMDB: 7475 (aligned on the cytoplasmic region).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.085.
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AUTHOR CONTRIBUTIONS

Y.-W.C., C.L.S., and L.A.R. prepared the samples. Y.-W.C. collected and processed the electron cryotomography data and generated the sub-tomogram averages. All authors analyzed and interpreted the electron cryotomography data. G.J.J. supervised the project. Y.-W.C., C.L.S., and G.J.J. wrote the paper, with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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